

Golgi-associated cPLA2 α Regulates Endothelial Cell–Cell Junction Integrity by Controlling the Trafficking of Transmembrane Junction Proteins

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In endothelial cells specifically, cPLA2 α translocates from the cytoplasm to the Golgi complex in response to cell confluence. Considering the link between confluence and cell–cell junction formation, and the emerging role of cPLA2 α in intracellular trafficking, we tested whether Golgi-associated cPLA2 α is involved in the trafficking of junction proteins. Here, we show that the redistribution of cPLA2 α from the cytoplasm to the Golgi correlates with adherens junction maturation and occurs before tight junction formation. Disruption of adherens junctions using a blocking anti-VE-cadherin antibody reverses the association of cPLA2 α with the Golgi. Silencing of cPLA2 α and inhibition of cPLA2 α enzymatic activity using various inhibitors result in the diminished presence of the transmembrane junction proteins VE-cadherin, occludin, and claudin-5 at cell–cell contacts, and in their accumulation at the Golgi. Altogether, our data support the idea that VE-cadherin triggers the relocation of cPLA2 α to the Golgi and that in turn, Golgi-associated cPLA2 α regulates the transport of transmembrane junction proteins through or from the Golgi, thereby controlling the integrity of endothelial cell–cell junctions.

INTRODUCTION

Endothelial cells form a monolayer lining the luminal surface of the entire vascular system. One of their main functions is to provide a semipermeable barrier between the blood and the underlying tissues. This barrier function is regulated to a great extent by endothelial adherens and tight junctions. The formation and the dynamic maintenance of these endothelial cell–cell junctions are crucial processes for the regulation of vascular homeostasis, and loss of junctional integrity is associated with many pathological disorders (van Nieuw Amerongen and van Hinsbergh, 2002).

Endothelial adherens junctions comprise the endothelial-specific transmembrane protein vascular endothelial (VE)-cadherin, whereas the transmembrane proteins occludin and endothelial-specific claudin-5 are part of the tight junctions (Bazzoni and Dejana, 2004). Like other transmembrane proteins, newly synthesized VE-cadherin, occludin, and claudins are transported through the secretory pathway to reach their final destination at the plasma membrane. One of the central organelles of the secretory pathway is the Golgi

apparatus. In mammalian cells, it is composed of stacked cisternae linked to one another to form the so-called Golgi ribbon (Mogelsvang and Howell, 2006). To date, very little is known about the trafficking of VE-cadherin, occludin, and claudin-5 from the Golgi to the junctions. Furthermore, it is unclear how the synthesis and the targeted transport of these junction proteins are regulated to sustain the formation, maturation, and dynamic maintenance of endothelial adherens and tight junctions in a timely manner. Growing evidence indicates that VE-cadherin and other adherens junction proteins are able to transduce long-lasting intracellular signals (Dejana, 2004). It is therefore possible that after their initial formation, adherens junctions transmit signals that regulate the synthesis and targeted transport of VE-cadherin and subsequently of tight junction components to their appropriate junctional location. In line with this idea, a recent elegant study demonstrated that VE-cadherin-mediated signaling directly controls the expression of claudin-5 and thereby the formation of tight junctions (Taddei *et al.*, 2008).

Phospholipases A2 (PLA2s) constitute a large family of enzymes that hydrolyze membrane phospholipids at the *sn*-2 position to generate free fatty acids and lysophospholipids (Schaloske and Dennis, 2006). On PLA2 enzymatic action, lysophospholipids locally accumulate in the membrane, thereby generating membrane curvature which contributes to the formation of transport carriers (Brown *et al.*, 2003; Zimmerberg and Kozlov, 2006). In this way, cytoplasmic PLA2s play a role in intracellular trafficking events as documented in several recent reports (Brown *et al.*, 2003). However, the identity of cytoplasmic PLA2s that participate

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Abbreviations used: cPLA2 α , cytosolic PLA2 alpha; HUVEC, human umbilical vein endothelial cell; MAFP, methylarachidonyl fluorophosphonate; PLA2, phospholipase A2; VE-cadherin, vascular endothelial cadherin.

in intracellular trafficking and the mechanism by which they regulate this process are still far from being understood.

Although best studied for its role in arachidonic acid generation (Leslie, 2004), the group IVA cytosolic PLA2 (cPLA2 α) has recently been implicated in the trafficking of a subset of proteins from the Golgi to the cell surface in epithelial cells (Choukroun *et al.*, 2000; Downey *et al.*, 2001). To access its phospholipid substrate, cPLA2 α translocates from the cytoplasm to membranes in a calcium-dependent manner (Clark *et al.*, 1991; Schievella *et al.*, 1995). In most cell types, increased intracellular calcium levels induce the translocation of cPLA2 α to the endoplasmic reticulum membrane, to the nuclear envelope, and to Golgi membranes (for review see Ghosh *et al.*, 2006 and references therein). The association of cPLA2 α to Golgi membranes occurs rapidly and transiently in response to cell stimulation by calcium-mobilizing agents such as ATP, thapsigargin, or the calcium ionophore A23187 (Evans *et al.*, 2001; Grewal *et al.*, 2003; Evans and Leslie, 2004).

In several endothelial cell types including human umbilical vein endothelial cells (HUVECs), cPLA2 α also translocates to the Golgi complex but with the unique property that this translocation occurs in response to cell confluence and is permanent, unless the cell monolayer is disrupted (Herbert *et al.*, 2005, 2007). Considering 1) the link between endothelial cell confluence and the formation of cell–cell junctions (Dejana, 2004), 2) the relationship between endothelial cell confluence and the Golgi association of cPLA2 α , and 3) the emerging role of cPLA2 α in intracellular trafficking, we hypothesize that Golgi-associated cPLA2 α plays a role in the transport of transmembrane junction proteins from the Golgi to the junctions, thereby regulating the formation and maintenance of endothelial cell–cell junctions.

After showing that the translocation of cPLA2 α to the Golgi occurs upon adherens junction maturation and before tight junction formation, we tested the contribution of cPLA2 α to the transport of transmembrane junction proteins from the Golgi to the junctions. To do so, we monitored the subcellular distribution of the transmembrane junction proteins VE-cadherin, occludin, and claudin-5 upon cPLA2 α depletion and upon inhibition of its enzymatic activity using various specific inhibitors.

MATERIALS AND METHODS

Antibodies and Inhibitors

Mouse anti-VE-cadherin clone 75 (Cat. No. 610252) and mouse anti-GM130 (Cat. No. 610823) were purchased from BD Transduction Laboratories (Lexington, KY). Rabbit anti-occludin (Cat. No. 71-1500) and mouse anti-claudin-5 (clone 4C3C2, Cat. No. 35-2500) were obtained from Zymed Laboratories (South San Francisco, CA). Mouse anti-VE-cadherin clone TEA 1/31 was obtained from Immunotech (Marseille, France; Cat. No. 1597). Mouse anti-tubulin (Ab-1) was obtained from Oncogene Research Products (Boston, MA; Cat. No. CP06). Mouse anti-cPLA2 α (Cat. No. sc-454) and goat anti-cPLA2 α (Cat. No. sc-1724) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). We confirmed the specificity of the goat anti-cPLA2 α by Western blotting (data not shown) as described by others (Grewal *et al.*, 2003; Herbert *et al.*, 2005, 2007). Affinity-purified rabbit anti-GM130 (ML07) was a kind gift from Martin Lowe (University of Manchester, United Kingdom) and has been characterized previously (Nakamura *et al.*, 1997). Alexa Fluor488-labeled donkey anti-goat, goat anti-mouse, goat anti-rabbit and Alexa Fluor555-labeled goat anti-mouse antibodies were obtained from Molecular Probes (Eugene, OR). Cy3-conjugated goat anti-rabbit antibody was from Jackson ImmunoResearch (West Grove, PA). Wyeth-1 and Pyrrolidine-2 (Pyr-2) were synthesized as described previously (Seno *et al.*, 2001; Ni *et al.*, 2006; Ghosh *et al.*, 2007). The commercial cPLA2 α inhibitor referred to as Pyrrolidine-CB (Pyr-CB) in this work was obtained from Calbiochem (La Jolla, CA; Cat. No. 525153). It is a pyrrolidine derivative characterized previously (compound 4d described in Seno *et al.*, 2000). Methylarachidonyl fluorophosphonate (MAFP) and bromoenol lactone (BEL) were obtained from Alexis Biochemicals (San Diego, CA). Indomethacin was obtained from Sigma (St. Louis, MO).

HUVEC Isolation and Culture

Umbilical cords were obtained from the Department of Obstetrics and Gynecology, Diaconessen Hospital, Utrecht, The Netherlands, with the informed consent of the parents. HUVECs were isolated from umbilical veins according to the method of Jaffe (Jaffe *et al.*, 1973). Cells were cultured on fibronectin-coated surfaces, in endothelial basal medium (EBM-2) supplemented with 2% fetal bovine serum, endothelial cell growth supplements EGM-2 (Cambrex, NJ), penicillin, streptomycin, and L-glutamine (Invitrogen, Carlsbad, CA). Cells were grown at 37°C in a 5% CO₂ humidified atmosphere and used between passages 1 and 4. To obtain sparse, subconfluent, and confluent cultures, HUVECs were seeded at 10,000 cells/cm² and used, respectively, 2, 5, or 7 d later. Medium was refreshed every 48 h. Postconfluent cells were incubated 3 more days after cells had reached confluence. Different seeding densities were used for RNA interference (RNAi) as indicated below. All experiments were reproduced at least three times using each time a different HUVEC isolation.

Treatment of HUVEC Monolayers

The VE-cadherin blocking antibody c175 was directly added (20 μ g/ml) into the culture medium of newly confluent HUVEC monolayers, and cells were incubated for 5 h at 37°C as described previously (Corada *et al.*, 2001) in a 5% CO₂ humidified atmosphere before being processed for immunofluorescence analysis. F-actin staining was used to control that c175 treatment was effective, namely that it induced stress fiber formation (Hordijk *et al.*, 1999). For treatments with the cPLA2 α inhibitors Wyeth-1, Pyr-2, Pyr-CB, and confluent or postconfluent cells were washed once with complete medium before inhibitors were applied in complete EBM-2 medium. Cells were incubated for 17 h with 5 μ M Wyeth-1, 2.5 μ M Pyr-2, 2.5 μ M Pyr-CB, or 0.05% DMSO (control). For treatment with MAFP, postconfluent cells were washed twice with serum-free medium and 10 μ M MAFP or 0.1% DMSO (control) was applied in serum-free EBM-2 containing all supplements for 17 h. Total lysates of treated cells were prepared in parallel with samples for immunofluorescent stainings in order to control the effect of the drugs on the expression levels of junction proteins (see *Cell Lysates Preparation*). For treatment with the cyclooxygenase inhibitor indomethacin, subconfluent or confluent cells were used. Cells were incubated for 17 h with 10 μ M indomethacin or 0.04% EtOH (control). For immunofluorescent experiments using postconfluent cells, stainings of junction proteins were also done at the start of the treatment to confirm that cells displayed adherens and tight junctions when the inhibitors were applied.

RNAi

HUVECs were seeded at 30,000 cells/cm² on fibronectin-coated 12-mm glass coverslips (for immunofluorescence) or on fibronectin-coated 24-well plates (for lysates preparation). Cells were grown for 36–48 h in complete EBM-2 growth medium with no antibiotics until they were 90–100% confluent. Cells were washed twice with OptiMEM (Invitrogen) and transfected with nontargeting small interfering RNA (siRNA; siCONT, D-001810-01, Dharmacon), cPLA2 α siRNA duplex 01 (sicPLA2 α #1, D-009886-01, Dharmacon) or cPLA2 α siRNA duplex 04 (sicPLA2 α #4, D-009886-01, Dharmacon Research, Boulder, CO). Transfection was performed with Oligofectamine (Invitrogen) using the manufacturer's instructions (300 nM oligonucleotides, 2 μ l Oligofectamine). After 4 h, EBM-2 with growth supplements but no antibiotics and containing 6% FBS was added to the cells. Cells were processed for immunofluorescence or lysates preparation 72 h after transfection. In these conditions, cells were not dedifferentiated because they expressed the endothelial-specific markers von Willebrand Factor and Endoglin. Silenced cells were still viable as confirmed by MTT viability assay. Note that at the time of transfection, adherens junctions were already formed, whereas tight junctions were not formed, as indicated by immunofluorescent staining of VE-cadherin and Claudin-5 (not shown). Under these experimental conditions, we therefore examined the effect of cPLA2 α silencing (over a period of 72 h) on the maturation/maintenance of adherens junctions and on the formation of tight junctions.

Immunofluorescence Microscopy

HUVECs grown on fibronectin-coated glass coverslips were fixed with 1% paraformaldehyde in HBSS for 15 min. Cells were permeabilized for 10 min with either 0.1% Triton X-100 (TX100) or 0.1% saponin, depending on the primary antibodies to be used subsequently (see *Results*). All steps subsequent to permeabilization with saponin were performed using PBS containing 0.1% saponin (PBS-sapo). Free aldehyde groups were quenched with 50 mM glycine in PBS or PBS-sapo. Cells were incubated sequentially for 1 h with primary antibodies diluted in PBS or PBS-sapo containing 1% BSA, except for the goat anti-cPLA2 α that was incubated overnight. Between antibody incubations, cells were washed three times for 10 min with PBS or PBS-sapo. They were incubated sequentially for 1 h with appropriate Alexa Fluor488-, Alexa Fluor555-, or Cy3-conjugated secondary antibodies diluted in PBS or PBS-sapo containing 1% BSA. The precise combination and working dilution of primary and secondary antibodies used for double labelings are available upon request. F-actin was stained with TRITC-conjugated phalloidin (Sigma). Nuclear staining was done with 4,6-diamidino-2-phenylindole (DAPI) di-

luted in PBS or PBS-sapo. Cells were mounted with ProLong Gold antifade Reagent (Molecular Probes). Images were acquired with an Olympus AX70 fluorescence microscope (Melville, NY) coupled to a CCD camera (Nikon DXM1200) using Nikon ACT1 software (Melville, NY). Acquisition settings were the same for different conditions within each experiment.

Cell Lysates Preparation

Cells plated in 24-well plates were treated with inhibitors or siRNA, in parallel to cells processed for immunofluorescence experiments. Cells were washed once with HBSS (PAA Laboratories, Linz, Austria) and incubated on ice for 5 min with lysis buffer (25 mM Tris HCl, pH 8, 1% TX100, 100 mM NaCl, 10 mM EDTA, 1 \times Complete EDTA-free protease inhibitor cocktail, and 1 mM Na₃VO₄). Cells were scraped, pooled from three wells, and centrifuged for 10 min at 13,000 rpm at 4°C. Supernatants were collected and protein determination was performed using a BCA protein assay kit (Pierce, Rockford, IL).

Western Blotting

Western blotting, immunoblot analysis, and membrane stripping were performed as described previously (Klapisz *et al.*, 2002). We loaded 5 μ g protein on 12% SDS-PAGE for the detection of claudin-5, and 15 μ g protein on 8% SDS-PAGE for the detection of other proteins. For immunodetection of cPLA2 α , the mAb (sc-454) was used. For immunodetection of VE-cadherin, we used clone TEA 1/31. Working dilutions and incubation times used for each primary antibody are available upon request.

Quantification and Statistical Analysis

For experiments with the VE-cadherin blocking antibody c175 (see Figure 2), the percentage of cells displaying Golgi-localized cPLA2 α in control and c175-treated HUVECs was quantified in three independent experiments by examining 250 cells chosen randomly. We also scored hundred isolated cells displaying no cell–cell contact with neighboring cells based on the F-actin staining. Results are expressed as mean \pm SD ($n = 3$). One way analysis of variance (ANOVA) followed by Student-Newman-Keuls Multiple Comparisons test was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). To quantify the relative amount of Golgi-localized cPLA2 α (Supplementary Figure S1), entire cells and Golgi regions from the corresponding cells were selected as regions of interest (ROI) using ImageJ software (<http://rsb.info.nih.gov/ij/>). Background was eliminated from images by subtracting 10% of the maximal pixel intensity from all pixel values (Herbert *et al.*, 2007). Total fluorescence in ROI was determined by multiplying mean pixel intensity by the surface area of the ROI. The relative amount of Golgi-associated signal was determined by dividing total Golgi fluorescence by total cell fluorescence. No pixel-saturated images were used for analysis. Results are expressed as mean \pm SEM (≥ 30 cells per condition). Unpaired *t* test was performed using GraphPad Prism.

RESULTS

cPLA2 α Is Recruited to the Golgi Apparatus upon Adherens Junction Maturation and before Tight Junction Formation

The confluence-dependent relocation of cPLA2 α from the cytoplasm to the Golgi complex (Supplementary Figure S1) occurs specifically in endothelial cells (Herbert *et al.*, 2005, 2007). The cell type specificity of this phenomenon raises the possibility that cPLA2 α relocation to the Golgi is related to an endothelial-specific cellular process associated with cell confluence, such as the formation of adherens and tight junctions.

To test this, we first examined whether the confluence-dependent recruitment of cPLA2 α to the Golgi correlated with the formation of adherens junctions, which was monitored by immunofluorescent staining of VE-cadherin. In sparse cultures, cells had not yet established cell–cell contacts, and VE-cadherin was not detected. In these cells, cPLA2 α was present in cytoplasmic punctate structures (Figure 1A), as described in other cell types (Bunt *et al.*, 1997). In subconfluent cultures, a subpopulation of cells displayed a strong continuous VE-cadherin labeling at sites of cell–cell contacts indicating that adherens junctions were being formed. In these particular cells, cPLA2 α was located at the Golgi (Figure 1A, arrowheads). However, in cells with only initial cell–cell contacts (as evidenced by a faint discontinu-

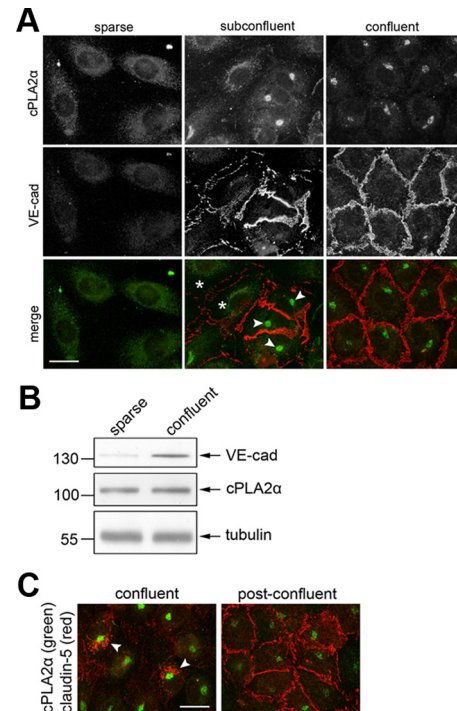


Figure 1. The confluence-dependent recruitment of cPLA2 α at the Golgi coincides with the maturation of adherens junctions and precedes the formation of tight junctions. (A) Sparse, subconfluent, and confluent HUVECs were processed for immunofluorescent staining of cPLA2 α and VE-cadherin (VE-Cad) using TX100 as permeabilizing agent. In the panel showing subconfluent cells, arrowheads indicate Golgi-associated cPLA2 α , and asterisks indicate cells where cPLA2 α is not associated with the Golgi. Note the difference in VE-cadherin staining intensity in these two cell populations, which indicates a difference in adherens junction maturity. Bar, 20 μ m. (B) Western blot analysis of cPLA2 α and VE-cadherin expression levels in cell lysates of sparse and confluent HUVECs. The membrane was reprobed with an anti-tubulin antibody to confirm equal protein loading. (C) Confluent and postconfluent HUVECs were processed for immunofluorescent staining of cPLA2 α (green) and claudin-5 (red) using TX100. Arrowheads indicate intracellular vesicular claudin-5 staining. Bar, 20 μ m.

ous VE-cadherin labeling), cPLA2 α was not associated with the Golgi (Figure 1A, asterisks). In confluent cultures, all cells exhibited a strong continuous VE-cadherin staining at sites of cell–cell contacts, indicating that adherens junctions were formed, and all cells displayed cPLA2 α at the Golgi (Figure 1A). Western blotting showed that the expression level of cPLA2 α had not changed when cells reached confluence, whereas VE-cadherin expression level increased (Figure 1B).

We then investigated the recruitment of cPLA2 α to the Golgi in relation to tight junction formation, monitored by immunofluorescent staining of endogenous claudin-5. In confluent cells, cPLA2 α was located at the Golgi as shown above, whereas claudin-5 was hardly detectable at sites of cell–cell contacts, indicating that tight junctions were not yet formed. In 10–15% of the confluent cells, claudin-5 was detected in intracellular vesicular structures (Figure 1C, arrowheads), possibly representing newly synthesized claudin-5 en route to the junctional complex. In postconfluent cells (3 d after confluence; see *Materials and Methods*), cPLA2 α was still located at the Golgi, and claudin-5 was detected at sites of cell–cell contacts in nearly all cells, indicating that tight junctions were formed (Figure 1C).

The Golgi localization of cPLA2 α was not affected by a refreshment of culture medium, nor was it by overnight serum deprivation (data not shown). Moreover, cPLA2 α was still localized at the Golgi up to 5 d after confluence was reached (data not shown).

Altogether, these data show that upon initial cell-cell contacts, cPLA2 α is not yet relocated to the Golgi and that some degree of maturity of adherens junctions is required to observe the relocation of cPLA2 α to the Golgi. Subsequently, tight junctions are formed, whereas cPLA2 α remains associated with the Golgi.

Disruption of Adherens Junctions with the Blocking anti-VE-Cadherin Antibody cl75 Reverses the Association of cPLA2 α with the Golgi

The previous data suggest that adherens junctions might transduce signals that sustain the long-term association of cPLA2 α with the Golgi. In turn, Golgi-associated cPLA2 α might contribute to the transport of junction proteins.

To test the first part of our hypothesis, we examined whether VE-cadherin is involved in regulating the association of cPLA2 α with the Golgi. We disrupted adherens junctions by treating newly confluent cells with the blocking anti-VE-cadherin antibody clone 75 (cl75; see *Materials and Methods*) that interferes with VE-cadherin homotypic adhesion (Corada *et al.*, 2001) and induces the formation of stress fibers (Hordijk *et al.*, 1999). As expected, cl75 efficiently disrupted adherens junctions, leading to the formation of intercellular gaps and even to the complete isolation of a small number of cells, as evidenced by F-actin staining (Figure 2A). In a majority of untreated cells ($67.3 \pm 2.3\%$, $n = 3$), cPLA2 α was localized at the Golgi, whereas only $29.6 \pm 2.1\%$ ($n = 3$) of cl75-treated cells displayed cPLA2 α at the Golgi. More strikingly, when isolated cells were specifically scored (identified by the absence of contact with neighboring cells), only $12.6 \pm 6.8\%$ ($n = 3$) of these cells displayed cPLA2 α at the Golgi (Figure 2, A and B). These data show that disruption of adherens junctions using the blocking anti-VE-cadherin antibody cl75 resulted in a dissociation of cPLA2 α from the Golgi. To rule out that this could primarily be a consequence of Golgi dispersion, GM130 was localized in cl75-treated cells. Figure 2C shows that cl75-treated cells in which cPLA2 α was not associated with the Golgi exhibited a normal distribution pattern of GM130, indicating that the Golgi was not dispersed. Altogether, these data suggest that cPLA2 α is maintained at the Golgi complex via a VE-cadherin-dependent mechanism.

Interfering with cPLA2 α Expression Alters the Integrity of Cell-Cell Junctions and Induces the Accumulation of Junction Proteins at the Golgi

We then tested the second part of our hypothesis, namely that Golgi-associated cPLA2 α facilitates the trafficking of transmembrane junction proteins from the Golgi to the junctions, thereby regulating the integrity of endothelial adherens and tight junctions.

We first examined the effect of cPLA2 α depletion on the integrity of adherens junctions by monitoring the subcellular distribution of the transmembrane protein VE-cadherin. Because of different antibody sensitivities to detergents, it was not possible to score the efficiency of cPLA2 α depletion at the single-cell level when double labeling of cPLA2 α and junction proteins was performed (Supplementary Figure S2). However, Western blot analysis showed that the two independent siRNA oligonucleotides (sicPLA2 α #4 and sicPLA2 α #1; see *Materials and Methods*) efficiently silenced cPLA2 α (Figure 3C). In cPLA2 α -depleted cells, the distribu-

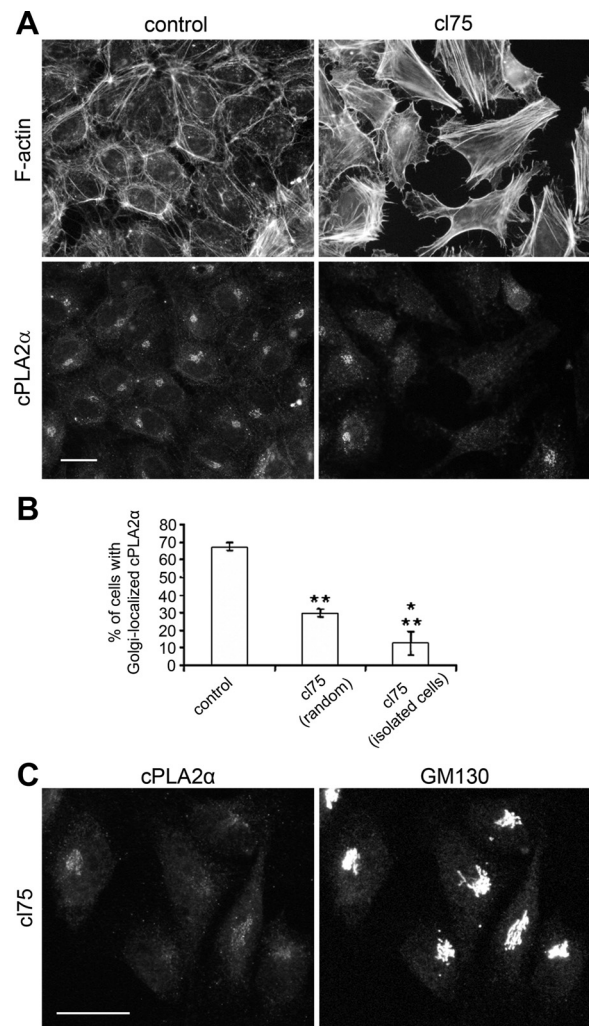


Figure 2. The blocking VE-cadherin antibody cl75 induces a relocation of cPLA2 α . (A) Newly confluent HUVECs were treated with anti-VE-cadherin-blocking antibody clone 75 (cl75, 20 μ g/ml, 5 h) or left untreated (control), fixed, and processed to detect cPLA2 α and F-actin. Bar, 20 μ m. (B) The percentage of cells displaying Golgi-localized cPLA2 α in control and cl75-treated HUVECs (left and middle column, respectively) was quantified by examining 250 cells chosen randomly. Hundred isolated cells displaying no cell-cell contact with neighboring cells were also scored (right column). Data are expressed as means \pm SD ($n = 3$). Values are: $67.3 \pm 2.3\%$ (control), $29.6 \pm 2.1\%$ (cl75, random), and $12.6 \pm 6.8\%$ (cl75, isolated cells). * $p < 0.01$ versus cl75 (random). ** $p < 0.001$ versus control. (C) Cells were treated for 5 h with 20 μ g/ml cl75, followed by immunofluorescent staining of cPLA2 α and GM130 using TX100. Note that in cl75-treated cells, the Golgi is not dispersed, whereas in most cells, cPLA2 α is dissociated from the Golgi. Microscope settings for the cPLA2 α staining are as in A. Untreated cells stained in parallel displayed colocalization of cPLA2 α and GM130, and the distribution of GM130 was identical to that in cl75-treated cells (not shown). Bar, 20 μ m.

tion of VE-cadherin was greatly disorganized at sites of cell-cell contacts (Figure 3A, arrowheads) compared with mock-depleted cells (transfected with a nonrelevant control oligonucleotide, siCONT). A strong intracellular vesicular VE-cadherin staining was observed in cPLA2 α -depleted cells but not in mock-depleted cells. Furthermore, VE-cadherin staining was observed in the Golgi area of most cPLA2 α -depleted cells as demonstrated by colabeling with

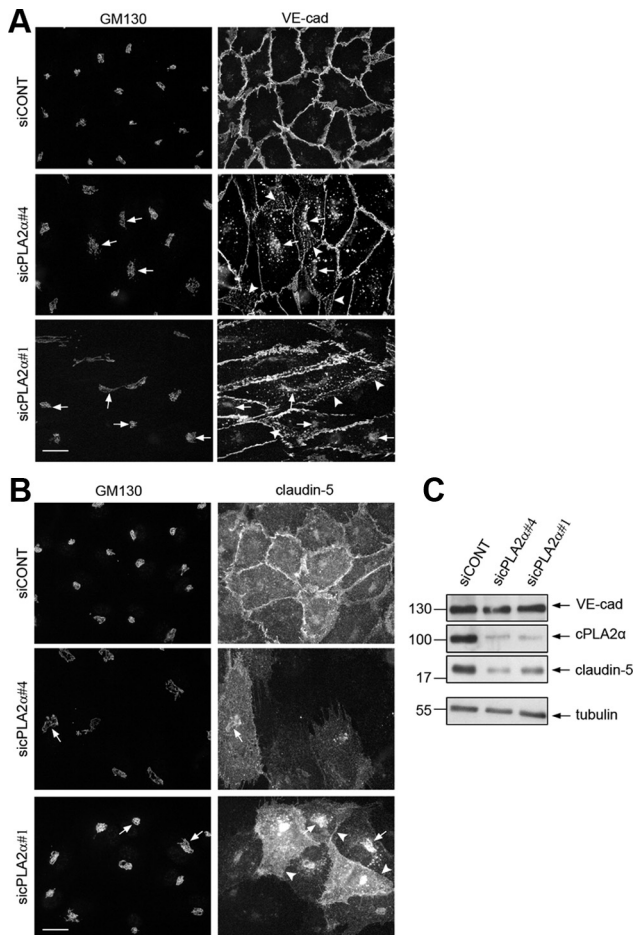


Figure 3. siRNA-mediated depletion of cPLA2 α alters adherens and tight junctions. (A) Nearly confluent HUVECs were transfected with two independent cPLA2 α -specific siRNA (siPLA2 α #4 or #1) or with a nonrelevant siRNA (siCONT). Note that adherens junctions were formed at the time of transfection (see *Materials and Methods*). After 72 h, immunofluorescent staining of GM130 and VE-cadherin (VE-Cad) was performed using saponin as permeabilizing agent. Arrowheads indicate highly disorganized areas of cell-cell contacts typically found in cPLA2 α -silenced cells. Arrows indicate the Golgi area of some siPLA2 α -silenced cells where VE-cadherin accumulates. Bar, 20 μ m. (B) Cells were processed as in A. Immunofluorescent staining of GM130 and claudin-5 was performed using saponin. Note that in siPLA2 α #1-transfected cells, cell-cell contacts display little claudin-5 (arrowheads). Arrows indicate the Golgi area of cPLA2 α -silenced cells where claudin-5 accumulates. Bar, 20 μ m. (C) Cells were treated as in A and B. Cell lysates were prepared and equal amounts of proteins were separated by 8 or 12% SDS-PAGE (see *Material and Methods*). Immunoblot analysis was performed to detect VE-cadherin, cPLA2 α , claudin-5, and tubulin as a loading control.

the *cis*-Golgi protein GM130 (Figure 3A, arrows). Western blot analysis of cellular extracts revealed a small decrease in VE-cadherin expression levels in siPLA2 α #4-transfected cells compared with mock-depleted cells and to siPLA2 α #1-transfected cells (Figure 3C), confirming that cPLA2 α depletion affected mostly VE-cadherin subcellular distribution rather than its expression level.

We next examined whether tight junctions were also affected by cPLA2 α depletion. Because tight junctions were not formed at the time of transfection (see *Materials and Methods*), we therefore evaluated the effect of cPLA2 α depletion

on their biogenesis. This was tested by immunofluorescent labeling of claudin-5 in cPLA2 α -depleted cells. At the single-cell level, cPLA2 α depletion led to a reduction of claudin-5 expression compared with mock-depleted cells, though the effect was more pronounced with siPLA2 α #4 than with siPLA2 α #1 (Figure 3B). Noteworthy, in siPLA2 α #1-transfected cells, neighboring cells that expressed claudin-5 displayed little amount of claudin-5 at sites of cell-cell contacts (Figure 3B, arrowheads). Furthermore, double labeling of claudin-5 and GM130 revealed a strong claudin-5 staining in the Golgi area of these cPLA2 α -depleted cells (Figure 3B, arrows). Western blot analysis of cellular extracts confirmed that claudin-5 expression levels were lower in cPLA2 α -depleted cells compared with mock-depleted cells, though the effect was more pronounced with siPLA2 α #4 than with siPLA2 α #1 (Figure 3C) as seen with immunofluorescence. Because claudin-5 was not expressed at the time of transfection (data not shown), it is likely that the lower expression levels of claudin-5 in cPLA2 α -depleted cells corresponds to a delay/block in claudin-5 biosynthesis. However, the accumulation of claudin-5 in the Golgi area of most cPLA2 α -depleted cells (using siPLA2 α #1) indicates a block in claudin-5 transport. Importantly, in the same conditions, the subcellular distribution of the nonjunctional transmembrane proteins ICAM-1 and endoglin was not affected by cPLA2 α depletion (Supplementary Figure S3).

Noticeably, the GM130 labeling pattern appeared different in cPLA2 α -depleted cells compared with mock-depleted cells (Figure 3, A and B). This was confirmed at the single-cell level by colabeling of GM130 and cPLA2 α (Supplementary Figure S4). This result indicates a change in Golgi organization upon cPLA2 α depletion.

Altogether, these data support the idea that cPLA2 α regulates specifically the transport of junction proteins in endothelial cells through or from the Golgi, thereby controlling junction integrity. However, the effect of cPLA2 α depletion on Golgi organization might account for the observed effect on junction integrity.

Inhibition of cPLA2 α Enzymatic Activity Affects the Integrity of Cell-Cell Junctions and Induces the Accumulation of Junction Proteins at the Golgi

To further investigate the role of Golgi-associated cPLA2 α in the regulation of adherens junction integrity and in tight junction formation, we next tested whether the enzymatic activity of cPLA2 α is required in these processes. We treated confluent cells with various specific cPLA2 α inhibitors and examined the subcellular localization of VE-cadherin, occludin, and claudin-5 by immunofluorescence. We used a commercially available specific cPLA2 α inhibitor (referred to as Pyr-CB) that is a pyrrolidine derivative (compound 4d described in Seno *et al.*, 2000) as well as two noncommercial but well-characterized specific cPLA2 α inhibitors, Pyr-2 and Wyeth-1, which do not inhibit iPLA2 nor secreted PLA2s as characterized previously (Seno *et al.*, 2001; Ono *et al.*, 2002; Ghosh *et al.*, 2007). Pyr-2 (also called pyrrophenone) and Pyr-CB are pyrrolidine derivatives with slightly different side chains. They are reversible, fast, and tight-binding inhibitors of cPLA2 α . They are active-site directed inhibitors and can penetrate intact cell membranes. Wyeth-1, which is structurally different from the pyrrolidine derivatives, is also a reversible, fast and tight binding inhibitor of cPLA2 α .

Treatment of confluent cells with Wyeth-1, Pyr-2, and Pyr-CB resulted in an increased intracellular VE-cadherin staining in the Golgi area (Figure 4A, top panels, arrows) as confirmed by double labeling with GM130 (not shown). The typical honeycomb-like pattern of VE-cadherin distribution

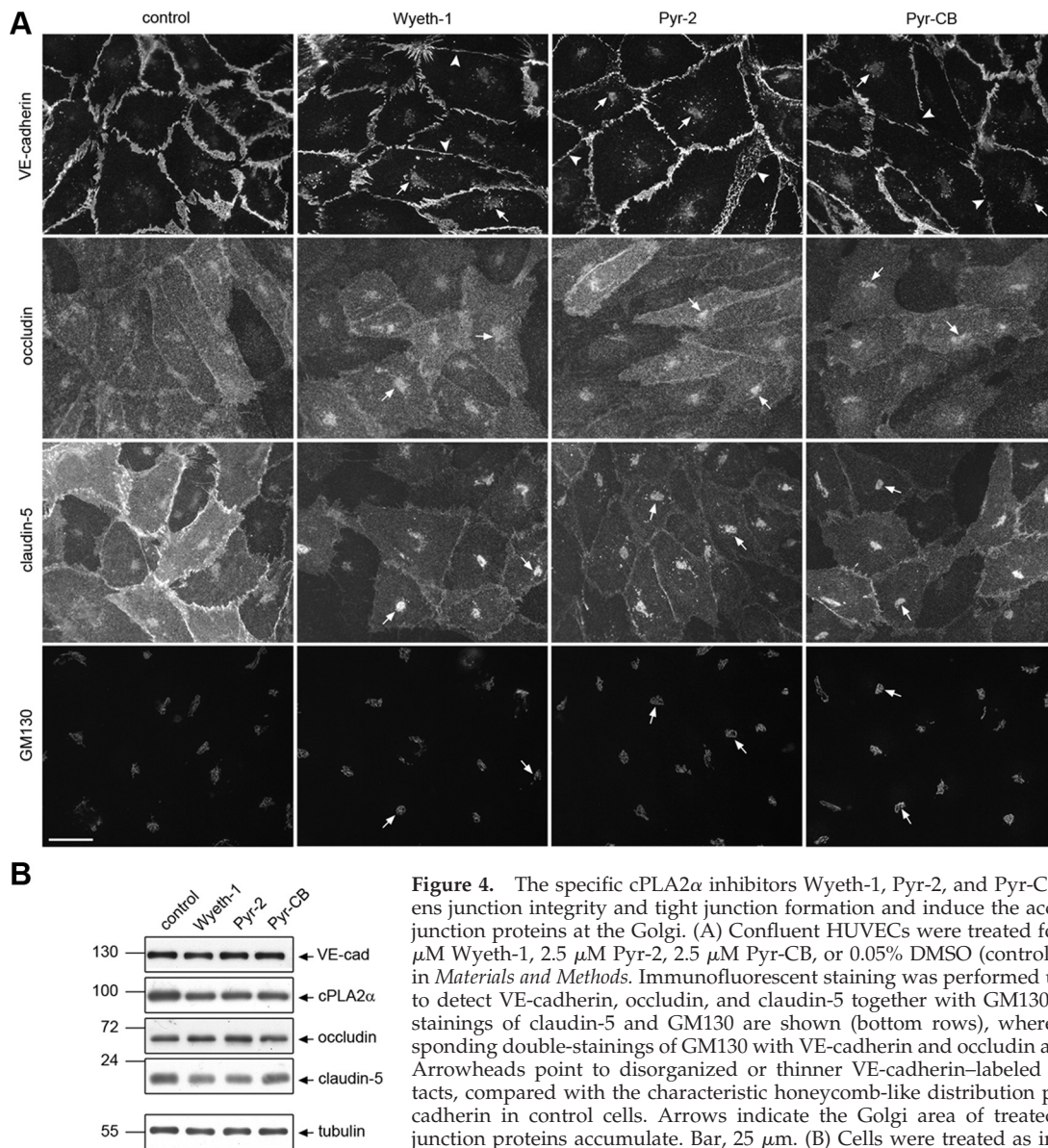


Figure 4. The specific cPLA2 α inhibitors Wyeth-1, Pyr-2, and Pyr-CB alter adherens junction integrity and tight junction formation and induce the accumulation of junction proteins at the Golgi. (A) Confluent HUVECs were treated for 17 h with 5 μ M Wyeth-1, 2.5 μ M Pyr-2, 2.5 μ M Pyr-CB, or 0.05% DMSO (control) as indicated in *Materials and Methods*. Immunofluorescent staining was performed using saponin to detect VE-cadherin, occludin, and claudin-5 together with GM130. The double-stainings of claudin-5 and GM130 are shown (bottom rows), whereas the corresponding double-stainings of GM130 with VE-cadherin and occludin are not shown. Arrowheads point to disorganized or thinner VE-cadherin-labeled cell-cell contacts, compared with the characteristic honeycomb-like distribution pattern of VE-cadherin in control cells. Arrows indicate the Golgi area of treated cells where junction proteins accumulate. Bar, 25 μ m. (B) Cells were treated as in A, and total lysates were prepared. Equal amount of proteins were separated by 8 or 12%

SDS-PAGE (see *Materials and Methods*). Immunoblot analysis was performed to detect VE-cadherin (VE-Cad), cPLA2 α , occludin, and claudin-5. Detection of tubulin was performed as a loading control.

at cell-cell contacts as seen in confluent cells (control) was disrupted in treated cells, in which cell-cell contacts appeared thinner, disorganized, and occasionally discontinuous (Figure 4A, top panels, arrowheads). The tight junction protein occludin was also found to accumulate in the Golgi area of most cells upon treatment with the cPLA2 α inhibitors (Figure 4A, arrows), as confirmed by double-labeling with GM130 (not shown). All three inhibitors induced a clear decrease of claudin-5 staining at cell-cell contacts, whereas cells displayed an increased staining of claudin-5 at the Golgi, as indicated by double-labeling with GM130 (Figure 4A, arrows). Importantly, the morphology of the Golgi apparatus of confluent cells was not altered by any of these inhibitors, as indicated by the unchanged distribution of GM130 (Figure 4A and Supplementary Figure S5). Also, the localization of cPLA2 α at the Golgi of confluent HUVEC was not altered by treatment with these inhibitors (Supplementary Figure S5).

Western blot analysis showed that treatment of confluent cells with the cPLA2 α inhibitors did not affect significantly the overall expression levels of cPLA2 α and of the junction proteins VE-cadherin and occludin, whereas claudin-5 expression levels were slightly reduced upon treatment (Figure 4B).

We also tested whether the enzymatic activity of cPLA2 α is required for the maintenance of cell-cell junction integrity after adherens and tight junctions were established. We treated postconfluent cells that had already established adherens and tight junctions, as indicated by the expression and distribution pattern of junction proteins at the start of treatment (data not shown). Both Wyeth-1 and Pyr-2 induced a decrease in VE-cadherin labeling at sites of cell-cell contacts and an increased labeling of occludin at the Golgi (Supplementary Figure S6A) as confirmed by colabeling of occludin with GM130 (Supplementary Figure S6B). The organization of the Golgi and the presence of cPLA2 α at the

Golgi were not affected by these inhibitors (Supplementary Figure S6C). Similar to the effects of Pyr-2 and Wyeth-1 on postconfluent cells, the less specific cPLA2 inhibitor MAFP also induced a decrease in VE-cadherin membrane staining, a decrease in the plasma membrane staining of occludin with a concomitant increase of occludin staining at the Golgi (Supplementary Figure S7A). The overall claudin-5 staining was decreased upon MAFP treatment, and a significant amount of cells displayed a strong staining of claudin-5 at the Golgi as indicated by double-labeling with GM130 (Supplementary Figure S7A, arrows). Western blot analysis confirmed the immunofluorescence data (Supplementary Figure S7B). Of note, when postconfluent cells were treated with BEL, the well-known inhibitor of the calcium-independent PLA2 group VI (iPLA2), the localization of the junction proteins studied remained unchanged (data not shown).

DISCUSSION

In this work we provide evidence that once recruited at the Golgi via a VE-cadherin-mediated process, cPLA2 α participates in the trafficking of junction proteins through or from the Golgi, thereby controlling endothelial cell-cell junction integrity. On cPLA2 α depletion, VE-cadherin- and claudin-5-targeted transport to cell-cell contacts was greatly affected, and both proteins were accumulated in the Golgi area. The inhibition of cPLA2 α enzymatic activity with specific inhibitors resulted in the decreased presence of adherens and tight junction proteins at cell-cell contacts and in their accumulation at the Golgi. This supports the notion that cPLA2 α activity is involved in the trafficking of junction proteins through or from the Golgi. Importantly, the effects of these inhibitors on junction protein distribution were not accompanied by a change in Golgi organization excluding the possibility that junction integrity was compromised as a result of Golgi disorganization. Also, the effects of the inhibitors cannot be attributed to a loss of Golgi localization of cPLA2 α . Altogether, these data show that the presence of an active cPLA2 α at the Golgi is critical for the transport of junction proteins.

cPLA2 α and the Regulation of Cell-Cell Junction Integrity

The confluence-dependent translocation of cPLA2 α to the Golgi is to our knowledge strictly endothelial-specific (Herbert *et al.*, 2007), suggesting that it is mediated by VE-cadherin, an endothelial-specific molecule of which expression and signaling function is closely linked to cell confluence (Dejana, 2004; Liebner *et al.*, 2006). This is supported by our data showing that the relocation of cPLA2 α to the Golgi correlates with a certain degree of maturity of adherens junction and that cPLA2 α dissociates from the Golgi when adherens junctions are disrupted by an anti-VE-cadherin antibody.

It will be interesting to determine whether the initial recruitment of cPLA2 α to the Golgi and/or its long-term association with the Golgi is regulated by VE-cadherin-mediated signaling. VE-cadherin clustering activates several signaling responses such as the activation of phosphatidylinositol-3-kinase (PI3K) and Akt (Taddei *et al.*, 2008). Interestingly, several studies have shown that PI3K activation is required for cPLA2 α activation, although this was demonstrated in nonendothelial cells (Silfani and Freeman, 2002; Myou *et al.*, 2003). Others have shown that the long-term association of cPLA2 α with the Golgi in confluent endothelial cells is calcium-independent and annexin A1-dependent (Herbert *et al.*, 2007). Annexin A1 becomes enriched at the Golgi in a confluence-dependent manner (Herbert *et al.*,

2007). A potential role of VE-cadherin-mediated signaling in this process has not been investigated.

Others have suggested that the association of cPLA2 α with Golgi membranes in confluent endothelial cells inactivates the enzyme, based on the fact that when cPLA2 α becomes associated with the Golgi, calcium-induced arachidonic acid release was greatly inhibited compared with nonconfluent cultures (Herbert *et al.*, 2005). We argue that this does not demonstrate that Golgi-associated cPLA2 α is enzymatically inactive, but rather shows that Golgi-associated cPLA2 α is less susceptible to calcium-induced activation. Using the same experimental system (confluent HUVECs), we show a clear effect of various specific cPLA2 α inhibitors on the trafficking of junction proteins through/from the Golgi. If Golgi-associated cPLA2 α was inactive, these inhibitors would not induce any effect. An important issue that will require further investigation is whether cPLA2 α is constitutively active on Golgi membranes or whether its activity is regulated, either from the cell surface via VE-cadherin-mediated signaling events or locally via a traffic-activated Golgi-based signaling pathway (Pulvirenti *et al.*, 2008).

Our findings relate to two important recent concepts. The first one emphasizes the importance of intracellular trafficking of junction components in the regulation of cell-cell junction integrity. However, to date, very little is known about the delivery of newly synthesized junction proteins to the membrane because most studies have focused so far on the role of endocytosis in the stabilization of junctions (Bryant and Stow, 2004; Xiao *et al.*, 2007; Yap *et al.*, 2007). The second concept is that VE-cadherin can transduce signals that regulate in a timely manner the formation and maintenance of adherens and tight junctions (Dejana, 2004; Liebner *et al.*, 2006). Experimental evidences for such cross-talk between VE-cadherin signaling and junction biogenesis/maintenance are mostly extrapolated from studies on epithelial E-cadherin (Braga and Yap, 2005; Mege *et al.*, 2006). However, one recent study has demonstrated a direct role of VE-cadherin-mediated adhesion/signaling in the control of claudin-5 expression (Taddei *et al.*, 2008), placing VE-cadherin as a master regulator of tight junction biogenesis and maintenance.

Our data raise the question as to whether cPLA2 α regulates claudin-5 expression as well as its trafficking. Indeed, cPLA2 α depletion resulted in a strong reduction in claudin-5 expression (Figure 3). This reduction in claudin-5 expression likely corresponds to a block in synthesis because at the time of transfection with siRNA, cells do not express claudin-5. In contrast the specific cPLA2 α inhibitors only mildly decreased claudin-5 expression when applied on confluent cells (Figure 4). The difference in these two approaches is that VE-cadherin distribution at cell-cell contacts was clearly more disrupted in cPLA2 α -depleted cells than in cPLA2 α -inhibited cells, possibly because of different lengths of treatment (72 vs. 17 h, respectively). Because impaired VE-cadherin adhesion/signaling induces the repression of claudin-5 expression (Taddei *et al.*, 2008), it is likely that the reduction in claudin-5 expression in our experimental settings is not directly regulated by cPLA2 α itself, but results from modifications in VE-cadherin junctional distribution.

On the basis of our findings, we propose a model (Figure 5) whereby VE-cadherin mediates a signaling pathway that triggers the association of cPLA2 α with the Golgi complex. In turn, Golgi-localized cPLA2 α participates in adherens junction maturation/maintenance by regulating the trafficking of VE-cadherin through/from the Golgi to the junctions. We also propose that at a later stage, once cells express the

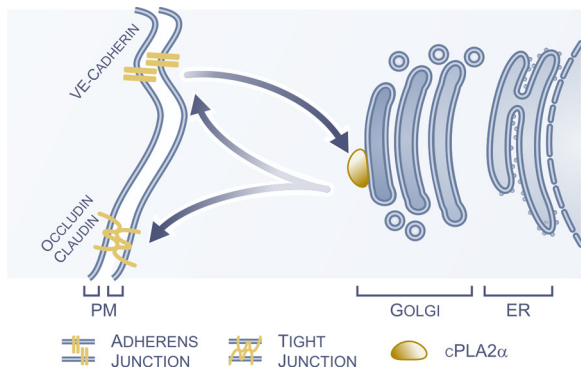


Figure 5. Model for the role of Golgi-localized cPLA2 α in the regulation of cell–cell junction integrity through the control of junction protein trafficking. On reaching confluence, endothelial cells make contact with one another through homotypic interactions of VE-cadherin. These initial cell–cell contacts mature into adherens junctions. When sufficient VE-cadherin molecules cluster at cell–cell contacts, they trigger a signaling pathway that results in and sustains the long-term relocation of cPLA2 α from the cytoplasm to Golgi membranes. In turn, once localized at the Golgi, cPLA2 α regulates the trafficking of VE-cadherin from the Golgi to cell–cell contacts, thereby contributing to the maturation/maintenance of adherens junctions. In time, cells start to produce tight junction proteins, such as occludin and claudin-5. Their transport from the Golgi to cell–cell contacts is also regulated by Golgi-associated cPLA2 α , which in this way contributes to the biogenesis/maintenance of tight junctions. This model illustrates the possibility of a cross-talk between VE-cadherin signaling and cPLA2 α -dependent intracellular trafficking. PM, plasma membrane; ER, endoplasmic reticulum.

tight junction proteins occludin and claudin-5, Golgi-associated cPLA2 α regulates their trafficking through/from the Golgi, supporting the formation and the maintenance of tight junctions.

cPLA2 α and Protein Trafficking through/from the Golgi

How does Golgi-localized cPLA2 α participate in the trafficking of proteins through/from the Golgi? As described above, the enzymatic activity of cPLA2 α is important for its role in trafficking. Two nonmutually exclusive mechanisms initiated by cPLA2 α enzymatic action could potentially regulate trafficking events. First, cPLA2 α could directly affect membrane curvature by producing inverted-cone-shaped lysophospholipids in one leaflet of the lipid bilayer (Brown *et al.*, 2003), thereby facilitating the formation of transport carriers. Second, through the release of arachidonic acid, cPLA2 α could influence transport processes by arachidonic acid-mediated regulation of the SNARE fusion machinery (Darios and Davletov, 2006; Davletov *et al.*, 2007). Furthermore, the arachidonic acid metabolites prostaglandin E2 and prostaglandin I2 (prostacyclin) are known to enhance the formation of endothelial adherens junctions via a cAMP-Epac-Rap1-signaling pathway on relatively a short time scale (minutes to 1 h; Fukuhara *et al.*, 2005). It is therefore possible that cPLA2 α regulates junction formation via a prostaglandin-dependent pathway. Although prostaglandin production is very low in confluent endothelial cells (Evans *et al.*, 1984; Whatley *et al.*, 1994; Herbert *et al.*, 2007), it might still be sufficient to elicit an autocrine cAMP-mediated response. We found that overnight treatment of subconfluent and confluent HUVECs with the cyclooxygenases inhibitor indomethacin (up to 10 μ M) did not influence the subcellular distribution of VE-cadherin and claudin-5 (data not

shown) as opposed to the observed effects with cPLA2 α inhibitors over the same period of time. This indicates that oxygenated metabolites of arachidonic acid do not play a role in the transport of junction proteins from/through the Golgi.

In line with our findings, previous work had established that cPLA2 α is involved in the delivery of a subset of transmembrane proteins to the cell surface of kidney epithelial cells (Choukroun *et al.*, 2000). Also, recent work demonstrates that cPLA2 α is involved in the intra-Golgi trafficking of two secretory proteins in HeLa cells (San Pietro *et al.*, 2009). These observations raise the questions as to whether the role of cPLA2 α in trafficking is cell type- and cargo-specific. Although the function of cPLA2 α has been extensively studied in many cell types (Ghosh *et al.*, 2006), its role in trafficking has so far only been evidenced in confluent endothelial cells (our work) and in epithelial cells (Choukroun *et al.*, 2000; Downey *et al.*, 2001; San Pietro *et al.*, 2009). Because both endothelial and epithelial cells form adherens and tight junctions and become polarized, cPLA2 α might be specifically involved in trafficking processes related to junction formation and/or to cell polarity. This idea also applies to the role of cPLA2 α in intra-Golgi trafficking in HeLa cells (San Pietro *et al.*, 2009). Although HeLa cells cultured in monolayer are nonpolarized and do not display tight junctions, these epithelial carcinoma cells do form tight junctions and polarize under specific growth conditions (Shimojo *et al.*, 1995; Dessus-Babus *et al.*, 2000). Because these cells have retained the capacity to polarize under specific circumstances, it is possible that they possess polarity-related modes of transport—such as cPLA2 α -driven trafficking—that are effective even in a nonpolarized cellular context.

Several findings support the idea that cPLA2 α regulates the trafficking of specific cargos. First, aged cPLA2 α –/– mice show a trafficking defect of aquaporin-1 and not of other aquaporins (Downey *et al.*, 2001). Second, in kidney epithelial cells, cPLA2 α overexpression abolished the basolateral delivery of the Na⁺-K⁺-ATPase α subunit, whereas it did not affect the basolateral localization of the Cl[–]/HCO₃[–] anion exchanger (Choukroun *et al.*, 2000). Our findings that the nonjunctional transmembrane proteins ICAM-1 and endoglin are normally trafficked in cPLA2 α -depleted cells, whereas the trafficking of VE-cadherin and claudin-5 is affected, also indicate a level of cargo specificity for cPLA2 α -regulated transport.

There is to date no experimental evidence showing that cPLA2 α knockout mice have altered endothelial junctions. It is possible that in these mice, another PLA2 compensates for the lack of cPLA2 α . It is also possible that the mouse and the human cPLA2 α play different roles. One argument in favor of this idea is that a patient displaying an inherited cPLA2 α functional deficiency (triple mutation S111P/R485H/K651R) suffers from small intestinal ulcerations that are much more severe than those observed in cPLA2 α knockout mice (Adler *et al.*, 2008). Thus functional inactivation of the cPLA2 α protein in human leads to more severe defects than knocking out the cPLA2 α gene in mice, pointing to species-specific functional roles of cPLA2 α .

cPLA2 α and the Control of Golgi Organization

We are the first, together with the recent work of San Pietro *et al.*, (2009), to address the role of cPLA2 α in the control of Golgi organization using specific cPLA2 α inhibitors of different structural classes, in combination with specific knock-down of the enzyme by siRNA. Previous work reporting that PLA2 inhibition induces the fragmentation of the Golgi used several PLA2 inhibitors that are not specific for cPLA2 α

(de Figueiredo *et al.*, 1999; Kuroiwa *et al.*, 2001). We found that the depletion of cPLA2 α induces a change in the morphology of the Golgi ribbon. This is in line with other studies suggesting the involvement of cPLA2 α in the maintenance of the Golgi structure (Choukroun *et al.*, 2000; Grimmer *et al.*, 2005; San Pietro *et al.*, 2009). In the present study we show that the Golgi morphology was affected when cPLA2 α was depleted (Supplemental Figure S4) but not when confluent or postconfluent cells were treated with specific cPLA2 α inhibitors (Figure 4; Supplemental Figures S5 and S6). The reason why the Golgi integrity was disrupted upon cPLA2 α depletion and not when cPLA2 α activity was inhibited in the context of confluent monolayers is still unclear. However, we consistently observed that in cPLA2 α -silenced cells, the Golgi structure was more disorganized when cells were subconfluent (unpublished findings). We also found that when nonconfluent sparse HUVEC cultures were treated overnight with cPLA2 α inhibitors, the Golgi organization was disrupted (unpublished findings). Because inhibition of cPLA2 α did not affect Golgi integrity in the context of nonproliferating cells (confluent and postconfluent cells), whereas the Golgi morphology was disrupted when cPLA2 α inhibitors were applied on proliferating cells (sparse cells, unpublished findings), it is possible that cPLA2 α is involved in the maintenance of the Golgi integrity in a cell cycle-dependent manner.

The fact that in confluent and postconfluent cells cPLA2 α inhibitors affect the trafficking of transmembrane junction proteins without affecting the Golgi organization suggests that the role of cPLA2 α in the maintenance of Golgi organization and its role in regulating the transport of proteins through/from the Golgi are independent processes and are probably regulated by different mechanisms.

In conclusion, our study illustrates a novel role for cPLA2 α in the control of endothelial cell–cell junction integrity, through the regulation of junction proteins transport through/from the Golgi. Further investigation is needed to elucidate the mechanism by which cPLA2 α regulates this transport and to clarify at which level of the Golgi this enzyme is acting.

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